

The effects of subdiffusion on the NTA size measurements of extracellular vesicles in biological samples

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Abstract

The interest in the extracellular vesicles (EVs) is rapidly growing as they became reliable biomarkers for many diseases. For this reason, fast and accurate techniques of EVs size characterization are the matter of utmost importance. One increasingly popular technique is the Nanoparticle Tracking Analysis (NTA), in which the diameters of EVs are calculated from the measurements of their diffusion constants. The crucial assumption here is that the diffusion of EVs follows the Stokes-Einstein relation, i.e. that the Mean Square Displacement (MSD) of a particle grows linearly in time ($\text{MSD} \propto t$). In this letter we show that this relation is severely violated in biological samples. In fact, at intermediate time periods MSD shows strongly sub-diffusive behavior ($\text{MSD} \propto t^\alpha$, $0 < \alpha < 1$) and for the longer periods it is affected by the boundary effects. The sub-diffusive behavior is well known in statistical physics, but most of its theoretical models do not provide the direct relation between the size of a particle and the generalized diffusion constant. We solve this problem by introducing the logarithmic model of sub-diffusion, aimed at retrieving the size data. In result we propose a novel protocol of NTA data analysis that accounts for the anomalous diffusion effects. Incorporating this aspect into analysis results in the change of the average EV diameter by 45% in comparison to the normal diffusion models. To corroborate

our analysis, we compare our results with the AFM studies of the same samples. Our methodology significantly reduces the discrepancy between the results obtained from NTA and direct imaging techniques.

Introduction

Extracellular microvesicles (EVs) are recognized as fragments of cell membranes generated by both prokaryotic and eukaryotic cells.¹ Most commonly used electron microscopy visualization allows to determine their diversity in diameter between 50 nm and 1000 nm.² Despite the small size, their presence was firstly reported using haemocytometry methods in late 60's and suggested that these lipid-rich particles originating from the osmophilic platelet granules ("platelet dust") have procoagulant potential.³ In the next twenty years, cell derived microparticles were almost neglected, refreshing to attract scientists' attention as possible thrombotic activators in late 90s.⁴ Microscopically, they consist of cell components including a lipid bilayer, cytoplasm and ribosomal fragments.⁵ It has been commonly accepted that EVs can be produced in two alternative secretory pathways: by budding cell membrane fragments - ectosomes, and by subsequent exocytosis.^{2,6,7} In blood, the main source of circulating EVs are platelets and endothelial cells, nevertheless in stressing conditions (hypoxia, inflammation or hyperglycemia) also macrophages and

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neutrophils can release a number of EVs.^{8,9}

Undoubtedly, the rapidly emerging research on EVs has a significant impact on the clinical and basic science, providing the novel potential for nanomedicine with new biomarkers, therapeutic targets and cell-to-cell communication vehicles.¹⁰ The majority of clinical data, obtained from the flow cytometry studies, show the heterogeneousness of EV surface antigen profile.^{1,4,8,9} However, the significant progress in nanotechnology brings a systemic approach to isolation, enumeration and the characterization of EVs size, shape and molecular components.^{11,12}

Resent research has demonstrated that, the larger population of EVs - ectosomes - represents rather heterogeneous population of vesicles whose size ranges from 0.1 to 1 μm in diameter.^{11,13} Their size distribution can be determined with different methods including scanning and transmission electron microscopy (EM),^{5,8,14} tunable resistive pulse sensing (TRPS),¹⁵⁻¹⁷ atomic force microscopy (AFM)¹⁸ and nanoparticle tracking analysis (NTA).^{19,20} Fig. Figure 1 illustrates their application to EV studies.

NTA is of particular interest in this letter. In this technique the sample of biological fluid is observed through a microscope. The laser beam shines on the sample from the direction perpendicular to the optical axis. The particles diffusing in the plane of the laser beam are visible thanks to the scattered light and their trajectories are recorded (Fig. Figure 1A). Then, for each trajectory the mean square displacement (MSD) is calculated. The Stokes-Einstein theory of diffusion predicts that MSD grows linearly in time, i.e.:

$$\begin{aligned} \text{MSD}(t) &= D_1 t \\ D_1 &= \frac{2\varepsilon k_B T}{3\pi\eta_0 d} \end{aligned} \quad (1)$$

where ε is dimensionality ($\varepsilon = 2$ for NTA), k_B is the Boltzmann constant, T is temperature, η_0 is the viscosity of a sample and d is the diameter of a particle. Equation (Eq. (1)) relates the diameter d of EV to the diffusion constant D_1 . Thanks to this dependence, it is possible to find the diameter distribution from the set of trajectories. The diffusion described by relation (Eq. (1)) is called *normal*.

Unlike AFM or EM, NTA does not require any

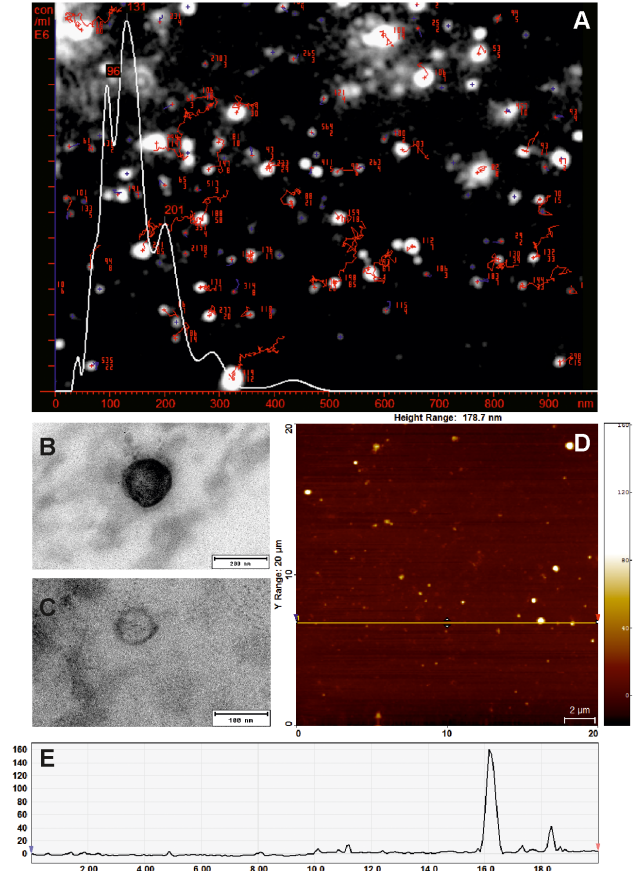


Figure 1: Extracellular vesicles visualization methods. (A) Image showing typical tracks of EVs moving under Brownian motion recorded by means of the NTA method. (B,C) Transmission Electron Microscopy (TEM) images of EVs isolated from human plasma using the ultracentrifugation method. Samples were fixed with 2.5% glutaraldehyde in 0.1M cacodylic buffer and then postfixed in 1% osmium tetroxide solution. (D) Topography of EVs placed on a poli-L-lysine coated slide. Samples were analyzed by means of Atomic Force Microscopy (AFM). The yellow line shows the cross-section, the colorimetric scale indicates the Z dimension presented as a height profile across the section line (E).

additional preparation of the sample (except for dilution) that could affect the biological state of EVs, which is its important advantage. It is also relatively cheap and fast, thus it is indicated as a 'method-of-choice' in the EVs characterization¹⁸ with a possibly broad use in diagnostics. However, the applicability of NTA to the biological samples requires further evaluation. The main problem is that for the same samples the distribution of EVs size obtained by NTA is significantly shifted towards the higher values of d , as compared to the direct imaging techniques (AFM, EM).²¹ This discrepancy occurs in the study of e.g. EVs from human placenta,²¹ but also for the coated gold nano-particles²² and, to a minor extend, model polystyrene beads.²⁰ Our measurements of EVs from human plasma, which we will discuss further, indicate that this discrepancy can be as high as 3-4 times the average EV diameter.

A few effects that cause this difference are already recognized. One important reason is that while NTA measures the hydrodynamic radius, AFM and EM measure the inelastic core of the particles. This fact explains why the discrepancy is lowest in the measurements of model beads.²⁰ Another reason is that NTA has low sensitivity to the small ($d < 50\text{nm}$) EVs, which cuts out the low-end of the size distribution.^{18,21} Yet another thing is the broadening of distribution due to the finite trajectory lengths,^{23,24} which becomes especially severe in the poly-disperse samples, when short trajectories are taken into account.²⁵ However, all of these effects might not be sufficient to fully explain the reported discrepancy.

In this letter we discuss yet another reason for the overestimation problem, which is especially important in the biological samples and has a more fundamental nature. Namely, we observe the violation of the Stokes-Einstein relation (Eq. (1)), i.e. the occurrence of the sub-diffusion, in which MSD grows sub-linearly with time. As we will exemplify with our measurements of EV size in human plasma, the application of the sub-diffusive models in the data analysis can significantly improve the coherence of NTA results versus the direct imaging techniques, such as AFM.

The NTA measurements are based on the observation of the diffusive motion, which in biological fluids might be affected by a plethora of

effects. These are e.g. the biochemical interactions with other EVs and proteins,^{9,26} unspecific excluded volume interactions,^{27,28} counter-sedimentation flows in the apparatus, inhomogeneous distribution of particles and the boundary effects, to mention a few. All of these issues violate the assumptions of the Stokes-Einstein theory²⁹⁻³¹ (Eq. (1)), which describes the freely diffusing particle in an uncorrelated, molecularly homogeneous environment. In what follows, the experimentally measured MSDs can deviate significantly from the linear dependence $\text{MSD} \propto t$. This can be seen in Fig. Figure 2A, where we show $\text{MSD}(t)$ for three exemplary trajectories from our measurements. For the short-to-intermediate time $\text{MSD}(t)$ resembles the free diffusion behavior, but the slope of the data on the log-log plot is clearly sub-linear, i.e. $\text{MSD}(t) \propto t^\alpha$, where $0 < \alpha < 1$. This is characteristic for sub-diffusion. From Fig. Figure 2A one can see that fitting the normal diffusion model to the sub-diffusive data leads to a significant underestimation of D_1 and, in result, an overestimated d . The free-diffusion regime is usually culminated with a local maximum, after which $\text{MSD}(t)$ decreases and fluctuates strongly. This behavior usually indicates that the diffusion begins to be dominated by the boundary effects.

The sub-diffusion (or, more generally, *anomalous* diffusion) is a phenomenon ubiquitously observed in the biological systems,³²⁻³⁴ but its recognition beyond the field of statistical physics is somewhat limited, especially in the bio-medical sciences. This is particularly unfavorable, since sub-diffusion affects the efficiency of transport in the molecularly crowded environments, e.g. cytoplasm.³² Microscopically, sub-diffusion is usually caused by the trapping events that intervene with the normal diffusion or the highly inhomogeneous viscosity of the system.

On the other hand, the literature regarding the mathematical models of sub-diffusion is very rich.^{29-31,35-38} However, most of these models are preoccupied with obtaining the anomalous exponent α , i.e. reproducing the asymptotic dependence:

$$\text{MSD}(t) = D_\alpha t^\alpha \quad (2)$$

where the generalized diffusion constant D_α is usually assumed as a fitable parameter. Although

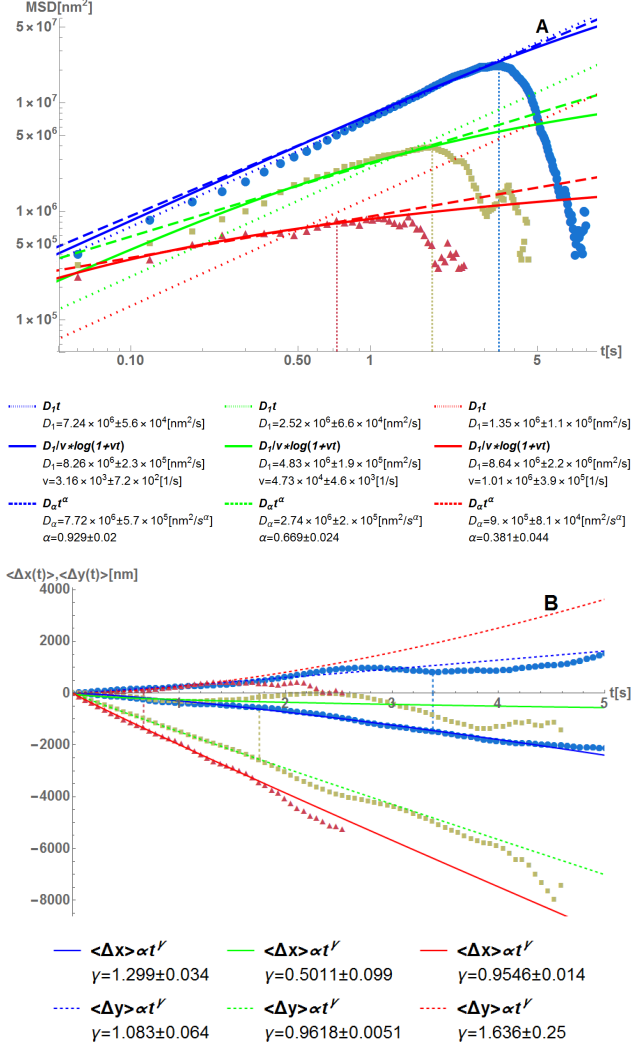


Figure 2: **A:** $\text{MSD}(t)$ plots for three exemplary trajectories, representing normal diffusion (blue circles), intermediate sub-diffusion (green squares) and strong sub-diffusion (red triangles). The dashed vertical lines indicate the cut-off time taken into analysis. The data has been fitted with linear model $\text{MSD}(t) = D_1 t$ (dotted lines), power-law model $\text{MSD}(t) = D_\alpha t^\alpha$ (dashed lines) and logarithmic model $\text{MSD}(t) = \frac{D_1}{v} \log(1+vt)$ (solid lines). For a well pronounced sub-diffusion the linear model is clearly deviating from the data (red and green plots), leading to the underestimation of diffusion constant. **B:** The mean position increment $\langle \Delta x(t) \rangle$ and $\langle \Delta y(t) \rangle$ for each trajectory from panel B. As previously, the dashed vertical lines indicate the cut off time. The increments are fitted with power-law model $\propto t^\gamma$.

(Eq. (2)) and (Eq. (1)) seem deceptively similar, they cannot be applied in the same manner. While D_1 is straightforwardly related to η_0 and d , there is no such simple dependence for D_α . In fact, one might interpret that $D_\alpha = D_1/\tau^{\alpha-1}$, where τ is the time-scale of sub-diffusion. However, while it is convenient to fit (Eq. (2)) to experimental data and recover the value of D_α , it is not possible to determine the unique values of D_1 and τ from the same procedure. In result, it is not possible to measure the distribution of diameters d from the model (Eq. (2)). Thus, for the purpose of application in NTA we must consider alternative approaches.

It should be emphasized that while the power-law model (Eq. (2)) is a popular description of sub-diffusion, it is mainly phenomenological. In fact, sub-diffusion is always a composition of multiple microscopic processes, as indicated by the Mori-Zwanzig type models³⁸ or by the ingenious viscoelastic approach, proposed by Goychuk.³⁶ Thus, different models of similar behavior can be justified on this basis. To remedy the problem of physical interpretation for D_α , we first attempted to use the modified power-law model: $\text{MSD}(t) = D_1 t / (1 + \frac{t}{\tau})^{1-\alpha}$, which asymptotically ($t \gg 0$) behaves like (Eq. (2)) and separates D_1 and τ . However, fitting this model to MSD data did not result in the stable values of D_1 and τ , similarly as in the case of (Eq. (2)). Most probably this is because NTA do not provide enough data on the $t \rightarrow 0$ behavior, for which these two models mainly differ.

Our final solution is provided by the logarithmic model. In the analysis section we will justify that each particle experience an approximately constant drift over the time interval of a measurement. Assuming that statistically this flow carries a particle from a less to more crowded region, we can introduce the time dependent viscosity $\eta(t)$ and approximate $\eta(t) \simeq (1 + vt)\eta_0$, where v is the speed of viscosity change, proportional to the constant drift. Assuming also that the short-time diffusion is still normal, the increment of MSD over some dt reads:

$$d\text{MSD}(t) = \frac{\epsilon k T}{3\pi\eta(t)d} dt \simeq \frac{D_1}{1+vt} dt \quad (3)$$

In result:

$$\text{MSD}(t) = \int_0^t ds \frac{D_1}{1 + \nu s} = \frac{D_1}{\nu} \ln(1 + \nu t) \quad (4)$$

The logarithmic model can mimic the power-law behavior over several orders of magnitude, when tuned properly. This model also preserves the standard interpretation of D_1 and it keeps D_1 and ν separated enough to allow reliable and repetitive MSD fits. One might also check that in this model $\lim_{t \rightarrow 0} \text{MSD}(t)/t = D_1$, so it predicts the non-zero diffusivity for every t , as desired. It also leads to the normal diffusion for $\nu = 0$, i.e. $\lim_{\nu \rightarrow 0} \text{MSD}(t) = D_1 t$. The quality of fits provided by this model is comparable to the power-law model.

Samples and measurements

Blood donors. Samples from 3 healthy donors (coded further as P1, P2 and P3) were used for this study. The samples has been collected in the conjunction with our previous study on diabetics.³⁹

Ethics. Bioethical Committee at Jagiellonian University Medical College (JUMC) accepted all project's protocols and forms, including an information for patients form and a consent form for participation in a research study. The permission No. KBET/206/B/2013 is valid until 31st of December 2017.

Blood collection and platelet poor plasma (PPP) preparation. All blood samples were drawn at the same time of the day (between 08:00 and 10:00 am) with venipuncture with > 21-gauge needle in the antecubital vein following the application of a light tourniquet. Citrate blood was centrifuged twice at 2500 g for 15 min to obtain PPP. The plasma samples were aliquoted and frozen at -80°C until further analysis. Before measurements samples were thawed in a 37°C water bath and vortexed for 30 s.

Nanoparticle Tracking Analysis (NTA) of plasma EVs To avoid EVs aggregation, all samples were diluted 100x in HEPES buffer (10 mM Hepes/NaOH, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4). The NTA measurement was performed with a NanoSight NS500 instrument (Malvern Instruments Ltd, United Kingdom), equipped with a

sample chamber with a 405-nm laser. The assay was performed at room temperature $23.3 \pm 0.1^\circ\text{C}$. For each donor (P1-P3) the measurements has been carried out 3 times. The samples were measured for 30s with the manual shutter and gain adjustments in advanced settings. The NTA 2.3 Build 0025 software was used for the capturing and preliminary analysis of the data.

Atomic Force Microscopy (AFM) analysis of plasma EVs. Poly-L-Lysine Slides from Thermo Scientific (cat no J2800AMNZ) were previously cleaved into $1 \times 1\text{cm}$ plates, rinsed both sides with distilled water and cleansed by compressed air. Samples with EVs were spread on glass slide and incubated for 1 hour in a humid chamber at room temperature. Then slides were rinsed three times by gently dipping in PBS (pH=7.4) and fixed in 2.5% glutaraldehyde in PBS for 30 minutes at room temperature. After that, slides were rinsed and analyzed by means of the AFM technique. To determine the size distribution of EVs, $20 \times 20\mu\text{m}$ topographical images of the samples were performed by Atomic Force Microscopy - Nanoscope IIIa Multimode-SPM (Veeco Instruments, Santa Barbara, CA, USA) in contact mode. AFM images were recorded in liquid (PBS) using fluid chamber and imaging conditions included a 0.4 Hz scan rate, 256 points collected per line (pixel resolution). As a probe, non-conductive pyramidal silicon nitride tip (MLCT, Bruker) with a resonance frequency 10-20 kHz, nominal spring constant 0.01 N/m and with a radius 100 nm was used. The topographical analysis of AFM images was carried out by using SPIP software version 6.5.2 (Image Metrology A/S, Horsholm, Denmark). To assess EVs diameter Particle and Pore Analysis mode was used with threshold set on 0.2 nm. Anything higher/brighter than the background was classified by algorithm as a particle. The size distribution histograms were prepared. Because the resolution of AFM images performed in contact mode depends on the diameter and shape of the tip (the tip convolution effect) and the tip used in this study was relatively wide, we also decided to recalculate the size of observed particles basing on correction method from Engel et al.⁴⁰

NTA data analysis

In the preliminary stage of analysis the NTA software identifies the trajectories of observed particles and this raw data are available to a user. Each trajectory is given as a sequence of positions $\vec{r}_i = (x_i, y_i)$ (in pixels) on the consecutive frames indexed by i , captured by the camera. Thanks to the *frame-rate* and *calibration parameter* provided by the software it is possible to recalculate these data into the actual physical units. NTA software is also able to distinguish the valid trajectories from the artifacts on the basis of combined length and scattered light intensity criteria. These trajectories are labeled as *included in the distribution* in the output *.csv* files. We restrict our analysis solely to these trajectories, but this is where we cease to use the NanoSight software for further analysis. The problem is that the exact operations applied to the data by the software are poorly documented, e.g. there is no information on how the trajectories are truncated and how exactly the drift is treated. Another problem is the application of the Finite Track-Length Adjustment (FTLA) algorithm, which is known to improve the accuracy of the mono-disperse sample measurements,²³ but has ambiguous influence on the polydisperse samples. E.g. it has been reported to cause artifacts¹² and in our case the FTLA and non-FTLA results differed significantly. Since our goal is to explicate the difference between the Stokes-Einstein and sub-diffusive models, we decided to perform the analysis on our own, maintaining the full control over the data processing.

Our first goal is to determine the MSD function individually, for each trajectory. The first step of our analysis is somewhat standard, we introduce the increments :

$$\Delta\vec{r}_i(n) = \vec{r}_{i+n} - \vec{r}_i \quad (5)$$

where $\Delta\vec{r}_i(n) = (\Delta x_i(n), \Delta y_i(n))$ and calculate their average value:

$$\langle \Delta\vec{r}_n \rangle = \frac{1}{N-n} \sum_{i=1}^{N-n} (\vec{r}_{i+n} - \vec{r}_i) \quad (6)$$

N is the maximal number of frames on which a particular molecule is recognized. $\langle \Delta\vec{r}_n \rangle$ is also

the measure of the average drift. Having found the increments, we can calculate the MSD(n), which reads:

$$\text{MSD}(n) = \frac{1}{N-n} \sum_{i=1}^{N-n} (\Delta\vec{r}_i(n) - \langle \Delta\vec{r}_n \rangle)^2 \quad (7)$$

In the Fig. Figure 2A, we present the log-log plots of MSD for three exemplary trajectories rescaled to the actual physical units. These plots show several features which are representative for our data. In general, MSD consists of an initial period of the linear growth on the log-log plot up to the local maximum, followed by the interval of decrease and strong fluctuations. Most of the free diffusion models, normal or sub-diffusion, cannot describe the decrease in MSD, which usually indicates the presence of boundaries in the system.³⁵ One possible exception is the Scaled Brownian Motion,⁴¹ which has a tendency to form a local maximum in MSD, but even in this case the effect is most pronounced in the confinement. Thus, we conclude that the post-maximum behavior is induced by the boundaries. For this reason, we must propose the criteria to exclude it from the further analysis based on the free diffusion models.

The features we just described are most representative for the long ($N > 9$) trajectories. Unfortunately, it is well known that while NTA measures a massive number of trajectories, there is no control over their length and only a mere 10 – 15% of them has the length of $N \geq 5$. Due to the poor statistics, the short trajectories result in the very noisy MSD plots, which might significantly deviate from what we described in the previous section. Additionally, the minimal trajectory length can also significantly broaden the diameter distribution, as discussed by Gardiner et. al.²⁵ In our case we face the same problem, but, in addition, we want to analyze only those trajectories for which the initial interval of MSD at least vaguely resemble the free-diffusion behavior. This decreases the number of trajectories even further. Thus, for the purpose of our study, a trajectory is accepted if its first three MSD points satisfy $\text{MSD}(1) < \text{MSD}(2) < \text{MSD}(3)$. This ensures the existence of a minimal, strictly growing sequence. This also means that we use trajectories of $N = 4$ at least, which is still close to the $N = 5$, recom-

mended by Gardiner. For donors P1, P2 and P3 the NanoSight software has admitted respectively 919, 679 and 366 trajectories of which 73%, 62% and 58% has been accepted according to our criterion.

We also had to decide on how many points of an individual MSD sequence are accounted for the free-diffusion behavior, i.e. we had to choose the truncation time T . Since we expect to encounter a local maximum in MSD, we propose that T is equal to the first n violating $\text{MSD}(n+2) > \text{MSD}(n)$. This condition can detect the systematic decrease in MSD, which usually occurs after the local maximum. However, it also allows some accidental detours from the strictly growing character of MSD (e.g. $\text{MSD}(n+1) < \text{MSD}(n)$ while $\text{MSD}(n+2) > \text{MSD}(n)$). The random variability of MSD must be expected, especially for short trajectories, since the sample size from which an individual value of $\text{MSD}(n)$ is calculated reads $N - n$. This number is usually low and decreases with subsequent n .

Having proposed the criteria of data selection and truncation, we address the central issue of this letter, which is the sub-diffusive dynamics of EVs. In Fig. Figure 2A we also show the fits obtained with the $\text{MSD}(t) \propto t$ and $\propto t^\alpha$ models for the exemplary trajectories. It is evident that in the case of strong sub-diffusion the linear model is insufficient and leads to the underestimated value of D_1 , and, in turn, to the overestimated d . Fig. Figure 3A shows the histogram of anomalous exponents α obtained from the fitting of model (Eq. (2)) to the truncated MSD functions. With the mean $\langle \alpha \rangle \simeq 0.70$, 0.69 and 0.71 for donors P1, P2 and P3 (respective standard deviations: 0.20 , 0.22 and 0.22) it is evident that most of EVs behave in the strongly sub-diffusive manner. However, as we discussed in the previous sections, the model $D_\alpha t^\alpha$ is ineffective, since the generalized diffusion constant D_α cannot be used to determine the distribution of diameters d .

Our final solution is based on the observation that each particle is carried by the flow of an approximately constant velocity. In the Fig. Figure 2B, we include the plots of $\langle \Delta x_n \rangle$ and $\langle \Delta y_n \rangle$ corresponding to MSD in panel A. Generally, $\langle \Delta \vec{r}_n \rangle$ evolves in a roughly linear manner for a prolonged period of time. We corroborated

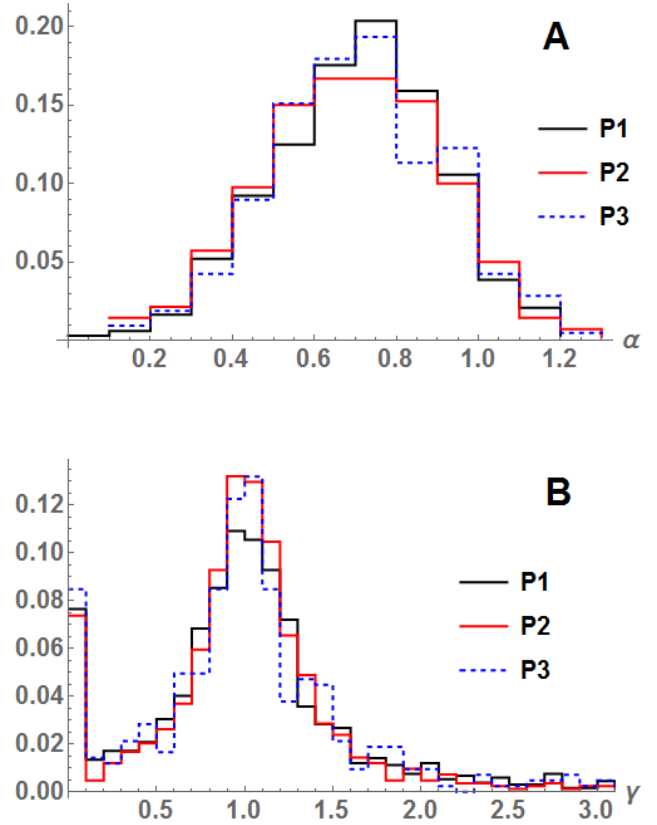


Figure 3: **A:** The histogram of anomalous exponent α , resulting from fitting the MSD data with the power law model, i.e. $\text{MSD}(t) = D_\alpha t^\alpha$. For each donor the histogram has a maximum near $\alpha \simeq 0.7$, which indicates the sub-diffusive behavior. **B:** The joint histogram of exponent γ resulting from fitting the average increments $\langle \Delta x_n \rangle$ and $\langle \Delta y_n \rangle$ with the power law model $\propto t^\gamma$. For all donors the distributions have distinct peaks at $\gamma \simeq 1$ and $\gamma \simeq 0$.

this idea by fitting the first T points of $\langle \Delta x_n \rangle$ and $\langle \Delta y_n \rangle$ with the power law model $\propto t^\gamma$. The histograms of γ (joined for x and y direction) are included in Fig. Figure 3B. For each donor the histograms have a distinct peak at $\gamma \simeq 1$, indicating a constant flow velocity. There is also additional peak at $\gamma \simeq 0$, related to the trajectories with $\langle \vec{r}_n \rangle = 0$.

Since statistically the drift can be seen as constant, we suppose the sub-diffusion is caused by the systematic growth in the viscosity perceived by a particle, i.e. $\eta(t) \simeq (1 + vt)\eta_0$. Qualitatively, particles are carried from less to more crowded area, which results in the sub-diffusion described by the logarithmic model (Eq. (4)). In Fig. Figure 2A we include the exemplary logarithmic fits to the MSD data. The quality of these fits is equivalent to the power-law model, but (Eq. (4)) can also account for the slight leveling of the data near the maximum. In Fig. Figure 4 we present the histograms of particle diameters d obtained with the use of the sub diffusion model (Eq. (4)) and the normal diffusion model. In both cases, fits were performed for the MSD data truncated in the same manner.

In Table Table 1 we compare the mean EV diameter $\langle d \rangle$ and its standard deviation obtained via every method and algorithm described in this paper. With the use of the sub-diffusion model for donors P1, P2 and P3 we obtained the mean values of d equal to 176nm, 113nm and 115nm. The respective standard deviations read 100nm, 83nm and 71nm. The same results for the normal diffusion model read 319nm, 214nm and 208nm with standard deviations 167nm, 154nm and 127nm. In comparison, the sub-diffusive model predicts the mean d which is $\simeq 45\%$ lower than the one predicted by the normal diffusion model. Similarly, the spread of data (measured by standard deviation) is also reduced by $\simeq 45\%$. These results clearly indicate that the diffusion model has a critical impact on the results of the NTA measurements.

To corroborate the sub-diffusive data, we also performed the AFM measurements of the same samples. In this case we obtained the respective mean diameters equal to 74nm, 66nm and 67nm with standard deviations 65nm, 54nm and 51nm. Obviously, these values are still roughly 40-50%

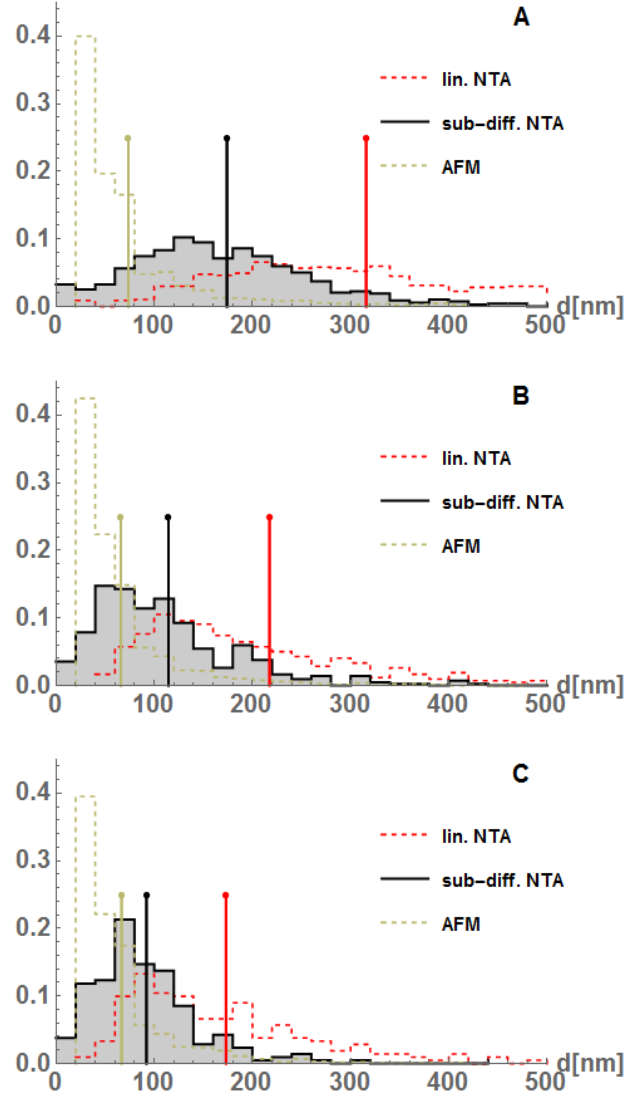


Figure 4: The histograms of EVs' diameter d for three donors (A is P1, B is P2, C is P3). The shaded black solid-line histogram is the distribution obtained via the sub-diffusive model ($\text{MSD}(t) = \frac{D_1}{v} \ln(1 + vt)$). Red dashed line is the histogram obtained for the normal diffusion model ($\text{MSD}(t) = D_1 t$) for the same data set. For comparison, the histogram of diameters obtained from AFM, for the same samples, are shown (dashed olive green). The vertical lines indicate the mean value for each distribution. Histograms are normalized to 1.

Table 1: The mean EV size $\langle d \rangle$ and its standard deviation for patients P1-P3 obtained from the NTA and AFM measurements for the same samples. The NTA data were analyzed with the normal diffusion model, our sub-diffusion model and the FTLA algorithm provided by the NanoSight software.

	$\langle d \rangle \pm \text{std. dev. [nm]}$			
	normal dif.	NTA sub-dif.	FTLA	AFM
P1	319±167	176±100	180±78	74±65
P2	214±154	113±83	159±66	66±54
P3	208±127	115±71	130±57	67±51

lower than the sub-diffusive results. However, this discrepancy can be attributed to the reasons listed in the introductory section, mainly the difference between the particle hydrodynamic radius and the size of its inelastic core as well as the influence of preparation on AFM samples.

As a final remark, let us mention that the results obtained via the sub-diffusive models are comparable to the distributions calculated by the NanoSight software with the FTLA algorithm. In this case the mean d reads 180nm, 159nm and 130nm for donors P1-P3, with standard deviations equal 78nm, 66nm and 57nm. However, the use of FTLA, which inherently assumes the normal diffusion model,²³ is questionable. The anomalous exponent histogram from Fig. Figure 3A clearly indicates that most of the trajectories have a sub-diffusive character. Nevertheless, this suggest that the combination of the sub-diffusive models and the FTLA approach could lead to a further improvement of the NTA method.

Summary

NTA is a technique of huge potential, but its broad application to the biological research is still in development and requires the clarification of many ambiguities. In this letter we analyzed the problem of anomalous diffusion affecting the results of NTA measurements in biological poly-disperse samples. We proposed the protocol of data processing and selection that allows us to stay in agreement with the requirements of the free-

diffusion model. We also introduced a simple model of sub-diffusion that can be ready-applied to retrieve the EVs size distributions. We showed that the application of sub-diffusion models instead of normal diffusion leads to the mean measured size of EV reduced by 45%, which is a significant difference. It also remedies the systematic discrepancies between NTA results and other techniques (such as AFM) for the same samples.

Acknowledgement Experiments have been financed by the Polish National Science Centre (NCN) grant No. 2012/07/B/NZ5/02510 (to E.S.). TEM images were performed by dr inż. Olga Woźnicka from the Department of Cell Biology and Imaging Institute of Zoology, Jagiellonian University.

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